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Anaerobic Central Metabolic Pathways in *Shewanella oneidensis* MR-1 Reinterpreted in the Light of Isotopic Metabolite Labeling▽

Yinjie J. Tang,[1,2] Adam L. Meadows,[2] James Kirby,[4] and Jay D. Keasling[1,2,3,4]*

Synthetic Biology Department, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California[1]; Department of Chemical Engineering, University of California at Berkeley, Berkeley, California[2]; Department of Bioengineering, University of California at Berkeley, Berkeley, California[3]; and California Institute for Quantitative Biomedical Research (QB3), University of California at Berkeley, Berkeley, California[4]

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It has been proposed that during growth under anaerobic or oxygen-limited conditions, *Shewanella oneidensis* MR-1 uses the serine-isocitrate lyase pathway common to many methylotrophic anaerobes, in which formaldehyde produced from pyruvate is condensed with glycine to form serine. The serine is then transformed through hydroxypyruvate and glycerate to enter central metabolism at phosphoglycerate. To examine its use of the serine-isocitrate lyase pathway under anaerobic conditions, we grew *S. oneidensis* MR-1 on [1-^{13}C]lactate as the sole carbon source, with either trimethylamine *N*-oxide (TMAO) or fumarate as an electron acceptor. Analysis of cellular metabolites indicated that a large percentage (>70%) of lactate was partially oxidized to either acetate or pyruvate. The ^{13}C isotope distributions in amino acids and other key metabolites indicate that under anaerobic conditions, although glyoxylate synthesized from the isocitrate lyase reaction can be converted to glycine, a complete serine-isocitrate pathway is not present and serine/glycine is, in fact, oxidized via a highly reversible degradation pathway. The labeling data also suggest significant activity in the anapleurotic (malic enzyme and phosphoenolpyruvate carboxylase) reactions. Although the tricarboxylic acid (TCA) cycle is often observed to be incomplete in many other anaerobes (absence of 2-oxoglutarate dehydrogenase activity), isotopic labeling supports the existence of a complete TCA cycle in *S. oneidensis* MR-1 under certain anaerobic conditions, e.g., TMAO-reducing conditions.

Shewanella oneidensis MR-1, formerly called *Shewanella putrefaciens* MR-1 or *Alteromonas putrefaciens* MR-1, is able to utilize many carbon sources, including lactate, acetate, pyruvate, and some amino acids (13, 21, 23, 26, 34, 36, 38). It can reduce a variety of electron acceptors besides oxygen, including Fe(III), Mn(IV), trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide, sulfur, nitrate, and fumarate (29, 32, 36). Over the last few decades, *Shewanella* spp. have been used in bioremediation applications involving a variety of toxic metals (21, 23, 26, 34, 36, 38). Recently, researchers also found that the versatile respiration ability of *S. oneidensis* MR-1 may be used to generate electricity from many substrates under anaerobic conditions (15–18, 28). Furthermore, *Shewanella* spp. are among the bacteria commonly implicated in the anaerobic spoilage of protein-rich foods, particularly marine fish (7, 10). The elucidation of the anaerobic pathways in *Shewanella* strains is therefore crucial for fully exploiting its potential in bioremediation and energy applications as well as improving existing methods of food preservation.

Traditionally, *S. oneidensis* (*S. putrefaciens*) strains (including MR-1) were thought to utilize the serine-isocitrate lyase pathway to produce phosphoenolpyruvate (PEP) from glyoxylate and formate during growth under anaerobic or oxygen-limited conditions (Fig. 1). This view was based on the very high activity of hydroxypyruvate reductase, a key enzyme in the serine-isocitrate lyase pathway, and it postulates the fixation of carbon by the condensation of formaldehyde with glycine to yield serine (dotted lines in Fig. 1) (8, 32, 40). Serine was thought to then enter the Embden-Meyerhof-Parnas (EMP) pathway and tricarboxylic acid (TCA) cycle through phosphoglycerate. This pathway is present ubiquitously in methylotrophic anaerobes (11); however, this pathway has not been rigorously verified experimentally for *S. oneidensis* strains.

For this study, we used *S. oneidensis* MR-1 as a model strain and grew it in the presence of [1-^{13}C]lactate under anaerobic conditions, with either TMAO or fumarate serving as the primary electron acceptor. The resulting ^{13}C labeling patterns of key metabolites, including several amino acids, pyruvate, and succinate, were determined using gas chromatography-mass spectrometry (GC-MS) (4, 5, 9). These labeling patterns permitted the identification of the active routes of carbon utilization in the main metabolic pathways of *S. oneidensis* MR-1. Interpreted in the light of reported enzymatic activities and transcriptome results as well as genome annotation information, the isotopic data from this study further extend our understanding of anaerobic carbon metabolism in *S. oneidensis* MR-1.

MATERIALS AND METHODS

Culture conditions. *S. oneidensis* strain MR-1 was purchased from the American Type Culture Collection (ATCC 700550) and stored at −80°C prior to use. All cultures used a previously reported minimal medium (34) containing the following (per liter of distilled water): 28 mM NH_4Cl, 1.34 mM KCl, 5 mM NaH_2PO_4, 0.7 mM Na_2SO_4, 1 mM $MgSO_4 \cdot 7H_2O$, 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 52 mM NaCl, 0.2 mM $CaCl_2$, and trace elements (1 liter of medium contains 10 mg $FeCl_2 \cdot 4H_2O$, 5 mg $MnCl_2 \cdot 4H_2O$, 3 mg $CoCl_2 \cdot 4H_2O$, 2 mg $ZnCl_2$, 0.5 mg $Na_2MoO_4 \cdot 4H_2O$, 0.2 mg H_3BO_3, 1 mg

* Corresponding author. Mailing address: Berkeley Center for Synthetic Biology, 717 Potter Street, Building 977, Mail Code 3224, University of California, Berkeley, CA 94720-3224. Phone: (510) 495-2620. Fax: (510) 495-2630. E-mail: keasling@berkeley.edu.

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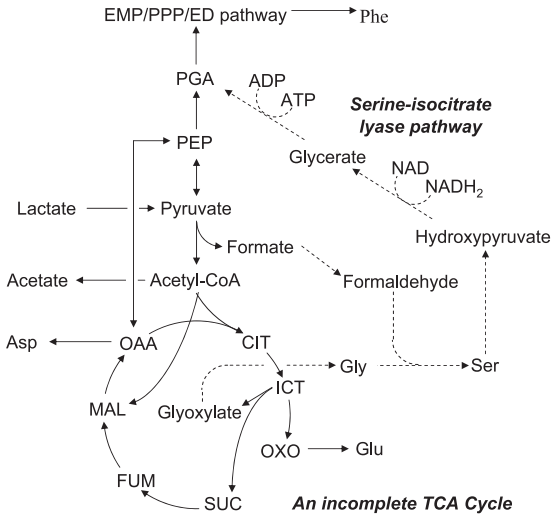

FIG. 1. *Shewanella oneidensis* MR-1 anaerobic carbon metabolism pathway. The solid arrows denote the central metabolic pathways, and the dotted arrows denote the serine pathway. The TCA cycle was proposed to be branched (e.g., OXO→SUC). Glycine was proposed to condense with formaldehyde to form serine, with serine then entering into TCA cycle reactions as oxaloacetate, consuming one $NADH_2$ and one ATP in the process. Abbreviations: CIT, citrate; ICT, isocitrate; MAL, malate; OAA, oxaloacetate; OXO, 2-ketoglutarate; PEP, phosphoenolpyruvate; FUM, fumarate; SUC, succinate; PGA, 3-phosphoglycerate; Glu, glutamate; Asp, aspartate; Gly, glycine; Phe, phenylalanine; C_1, 5,10-Me-THF (N^5,N^{10}-methylene-H_4 folate); EMP, Embden-Meyerhof pathway; PPP, pentose phosphate pathway; ED, Entner-Doudoroff pathway; TCA, tricarboxylic acid cycle. (Adapted from reference 32.)

$NiSO_4 \cdot 6H_2O$, 0.02 mg $CuCl_2 \cdot 2H_2O$, 0.06 mg $Na_2SeO_3 \cdot 5H_2O$, and 0.08 mg $Na_2WO_4 \cdot 2H_2O$). The medium was supplemented with ~20 mM 1-^{13}C-labeled sodium L-lactate (99%; Cambridge Isotope) as the carbon source and 30 mM TMAO (98% pure; Sigma) or 23 mM fumarate (99% pure; Sigma) as the electron acceptor. The medium pH was adjusted to 7.0 before filter sterilization. The MR-1 inocula for all experiments were grown aerobically in LB medium from a frozen stock (unlabeled culture) and then inoculated at 0.1% (vol/vol) into minimal medium containing [1-^{13}C]lactate in 50-ml Falcon tubes in an anaerobic hood with an atmosphere of 5% CO_2, 5% H_2, and 90% N_2 (Coy Laboratory Products Inc, Grass Lake, MI). The cultures were grown in the hood at 30°C without shaking and monitored by measuring the optical density at 600 nm (OD_{600}). The concentrations of lactate, acetate, pyruvate, and succinate in the culture medium (following centrifugation of the culture at 10,000 × *g* for 20 min at 4°C) were measured using enzyme assays (r-Biopharm, Darmstadt, Germany).

To prepare GC-MS samples by using bis(trimethylsilyl)trifluoroacetamide (BSTFA) as the derivatization reagent, 0.5 ml of supernatant was frozen in liquid nitrogen and then lyophilized overnight. The dried samples were prederivatized with a solution (0.3 ml) of 2% hydroxylamine hydrochloride (Fluka) in pyridine (Sigma-Aldrich) overnight at room temperature. Following this, each sample was derivatized at room temperature for 25 min, using 0.5 ml BSTFA (Sigma-Aldrich), before measurement by GC-MS. This derivatization added trimethylsilyl groups to carboxyls and converted oxoacids (e.g., pyruvate) to oximes for greater MS suitability. Decane (Aldrich Chemical) was used as an internal standard. Standards for the TCA pathway intermediates fumarate and succinate as well as pyruvate and glycine were prepared in the same way to generate standard curves for quantification. One microliter of the derivatized sample was injected into a gas chromatograph (Agilent model HP6890) equipped with a DB5-MS column (J&W Scientific, Falsom, CA) and analyzed using a mass spectrometer (Agilent model 5973). The column was held at 60°C for 1 minute after injection and then heated at 20°C/min to 130°C, 4°C/min to 150°C, and finally 40°C/min to 260°C, where it was held for 3 min. Helium carrier gas was used at a column flow rate of 1.2 ml/min, with a 1:20 split ratio at injection.

In order to measure the isotopomer labeling patterns in amino acids incorporated into cellular proteins, biomass was harvested by centrifugation at 10,000 × *g* for 20 min at 4°C and subsequently sonicated. The protein from the resulting lysate was precipitated using trichloroacetic acid and then hydrolyzed in 6 M HCl at 100°C for 24 h. The amino acid-HCl solution was dried under nitrogen flow overnight. GC-MS samples were prepared in 100 μl of tetrahydrofuran and 100 μl of *N*-(*tert*-butyldimethylsilyl)-*N*-*methyl*-trifluoroacetamide (Sigma-Aldrich). These samples were derivatized in a water bath at 70°C for 1 h, producing *tert*-butyldimethlysilyl (TBDMS) derivatives. One microliter of the derivatized sample was injected into the GC-MS equipped as described above. The GC column was held at 150°C for 2 min, heated at 3°C per minute to 280°C, heated at 20°C per minute to 300°C, and held for 5 min at that temperature. Two types of positively charged species were clearly observed by GC-MS (Fig. 2a and b), namely, unfragmented amino acids ([M-57]) and fragmented species ([M-159]) that had lost one carboxyl group (4). For amino acids containing two carboxylic groups, namely, aspartic acid and glutamic acid, the loss of the α-carboxyl group is strongly favored, as the amine group on the β-carbon allows the formation of an entropically stabilized fragment (6, 12). The two fragmented molecules ([M-57] and [M-159]) were used to determine if the α-carboxyl group was labeled. This study used the GC-MS data to determine the percentage of ^{13}C in each of the key metabolites. From these data, one can qualitatively investigate the metabolic pathways in the batch culture system.

RESULTS AND DISCUSSION

S. oneidensis MR-1 was grown in lactate minimal medium with either TMAO (OD_{600} = 0.08 at 48 h) or fumarate (maximum OD_{600} = 0.1 at 48 h) as the electron acceptor (Fig. 3 and Table 1). During growth, TMAO is reduced to trimethylamine, and fumarate is reduced to succinate. The observed minimum doubling time was approximately 8 h with TMAO and 6 h with fumarate. The major products of lactate metabolism were acetate and pyruvate, with over 70% of the lactate being converted to acetate, pyruvate, and succinate under both conditions. The addition of a small amount of unlabeled glycine (100 mg/liter) significantly enhanced the growth rate in the presence of both TMAO and fumarate, with the maximum OD_{600} exceeding 0.1 after just 24 h. Since the glycine concentration (~1.3 mM) was much lower than that of TMAO or fumarate, it is unlikely that the significant enhancement in biomass production was because the glycine served as an additional electron acceptor (i.e., glycine was reduced to acetate and ammonia by glycine reductase). In fact, glycine, an intermediate in the serine-isocitrate lyase pathway, may be employed as an electron donor and relieve some limitation in MR-1's anaerobic metabolism during growth in complete minimal medium (22, 34). Meanwhile, tracer experiments using glycine provided information on the direction of carbon flow through the serine pathway.

In order to investigate active anaerobic pathways, GC-MS was used to analyze the isotope distribution in key metabolites. Prior to analysis by GC-MS, pyruvate and succinate were derivatized with BSTFA, while key amino acids were derivatized with TBDMS. The presence of heavy isotopes other than ^{13}C in the carbon backbones of the molecules of interest was accounted for by following a published algorithm (14). The corrected mass fragment distributions of the metabolites of interest are listed in Tables 2, 3 and 4. The corrected, experimentally determined isotope distributions were used to identify the active metabolic pathways under anaerobic conditions.

Under TMAO-reducing conditions, pyruvate was almost entirely (96%) labeled at its carboxyl group, indicating that it was derived largely from the labeled lactate (Table 2). Proceeding around the TCA cycle, the majority (80%) of glutamate, which is derived from 2-ketoglutarate (OXO), was unlabeled. Of that

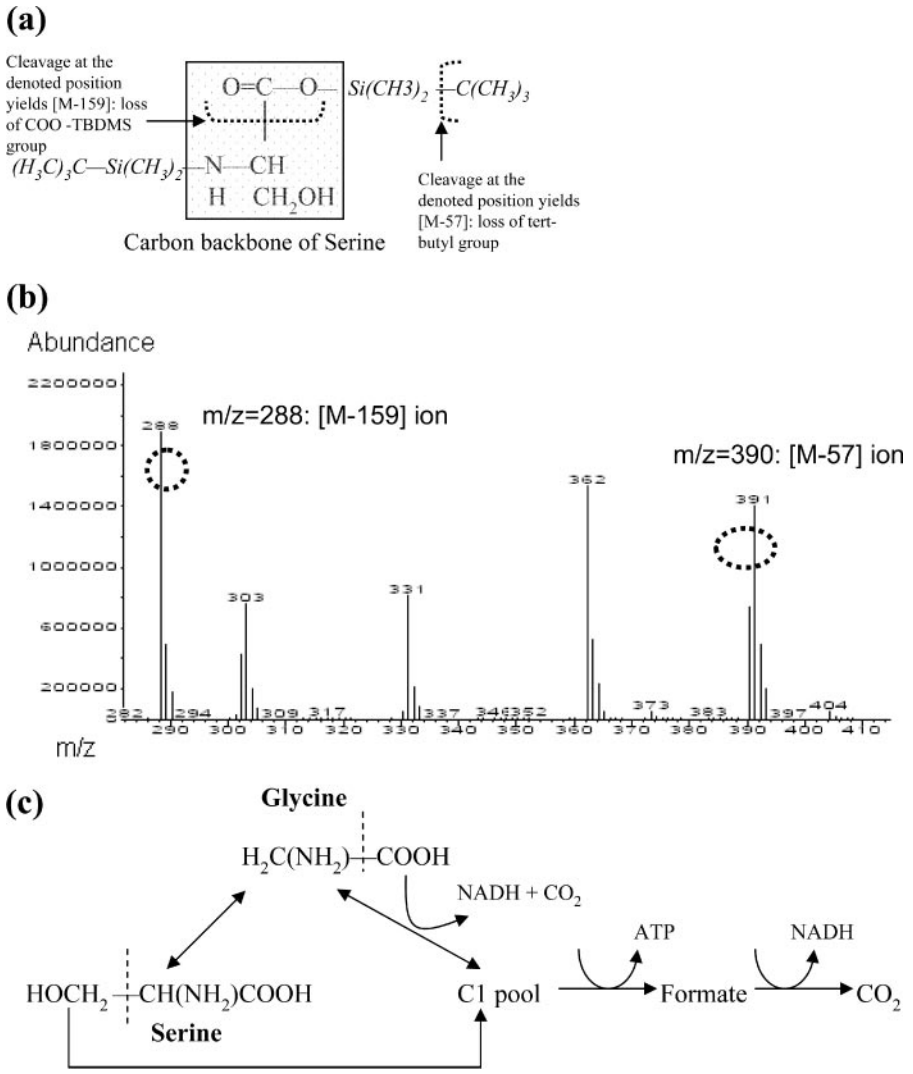

FIG. 2. Isotopic labeling evidence refutes the existence of the serine pathway proposed in Fig. 1. MS analysis was performed with TBDMS-derivatized serine from MR-1 hydrolysates grown under fumarate (23 mM) reduction conditions, with 100 mg/liter glycine added. (a) Fragments of TBDMS-derivatized serine. Derivatized groups are shown in italics. (b) MS spectrum of TBDMS-derivatized serine. The measured mass fragment series are *m/z* 390 (no loss) (M0 = 0.38, M1 = 0.61, and M2 = 0.01) and *m/z* 288 (loss of COO-TBDMS group) (M0 = 0.99). (c) Metabolic fates of serine and glycine in MR-1 (dashed lines indicate bonds where enzymatic action occurs). Serine is reversibly converted to glycine by serine hydroxymethyl-transferase. Glycine is oxidized to CO_2 + NH_4^+, producing NADH and C_1 (N^5,N^{10}-methylene-H_4 folate). C_1 can be oxidized to formate and then CO_2 to generate ATP and NADH.

which was labeled, most (~75%) molecules were labeled only at the α-carboxyl group, with much smaller percentages labeled only at the β-carboxyl group or at both carboxyl groups. Similarly, succinate, which can be formed by decarboxylation of 2-ketoglutarate or from isocitrate via isocitrate lyase, was largely (84%) unlabeled. This evidence indicates that most of the succinate did not come from oxaloacetate (OAA), because if the TCA cycle were incomplete and the left branch was running backwards (reductively) to supply succinate, succinate would be more highly labeled due to the need to make OAA from pyruvate. Of the succinate that was labeled, most (~69%) was labeled at a carboxyl group. This labeling pattern suggests that unlabeled succinate was produced via oxidation of 2-ketoglutarate (loss of the labeled α-carboxyl group as $^{13}CO_2$). The small amount of labeled succinate (16%) was probably derived via isocitrate lyase which labeled one of the carboxyl groups in succinate. This conclusion is consistent with previous work, which demonstrated activities of key enzymes in the TCA cycle (citrate synthase, isocitrate dehydrogenase, and 2-ketoglutarate dehydrogenase) and the presence of one of the key enzymes in the glyoxylate shunt (isocitrate lyase) under anaerobic conditions (32).

The majority of aspartate (58%) and methionine (64%) molecules, which are derived from OAA, were unlabeled. Of the aspartate and methionine molecules that were labeled, most were labeled at the α-carboxyl group. The reported activity of isocitrate lyase in *S. putrefaciens* is 1 to ~2 orders of magnitude lower than those of other key enzymes in the TCA cycle, so glyoxylate was assumed to be produced primarily for synthesis of glycine rather than malate (MAL) under anaerobic condi-

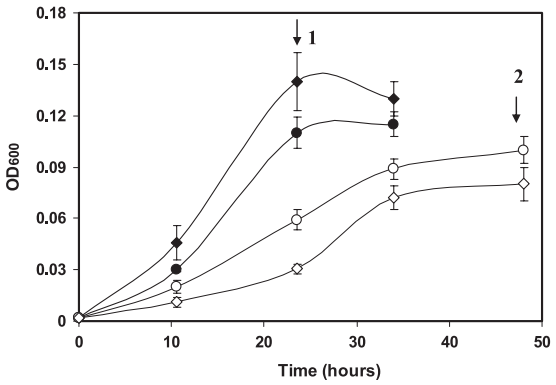

FIG. 3. Anaerobic growth curves for *Shewanella oneidensis* MR-1 in minimal L-[^{13}C]lactate medium. ○, growth under fumarate reduction conditions; ◇, growth under TMAO reduction conditions; ◆, growth with 100 mg/liter glycine under fumarate reduction conditions; ●, growth with 100 mg/liter glycine under TMAO reduction conditions. Arrow 1 was the sampling point for growth with glycine, and arrow 2 was the sampling point for growth without glycine.

tions (32). The labeling patterns in aspartate and methionine indicate that the unlabeled OAA pool was produced primarily from succinate through the TCA cycle instead of via the glyoxylate shunt. The labeled OAA pool was produced primarily from the anapleurotic reactions (PEP→OAA and pyruvate→MAL→OAA). These reactions may also incorporate a second labeled $^{13}CO_2$ (generated during pyruvate oxidation) as the β-carboxyl group in malate or oxaloacetate, which is then converted to aspartate.

The labeling patterns suggest the presence of the reactions 2-ketoglutarate→succinate→malate→oxaloacetate, which form a complete TCA pathway cycle under TMAO reduction conditions; however, under anaerobic conditions in many other bacteria (such as *Escherichia coli*), the carbon flow in the TCA cycle is branched (2-ketoglutarate dehydrogenase activity is absent), and succinate originates from the OAA pool (39). This difference can be explained by MR-1's unique respiratory system. Quinones, including ubiquinones and menaquinones, are essential components of bacterial respiratory chains (39). In *E. coli*, ubiquinones predominate in aerobic and nitrate-grown cells, whereas menaquinone predominates in fumarate- and TMAO-grown cells. On the other hand, MR-1 does not use menaquinone during TMAO reduction, but rather uses various ubiquinone species (19, 25, 39). Thus, TMAO reduction in MR-1 may be more like aerobic respiration than like anaerobic respiration, i.e., the TCA cycle is complete, as suggested previously for other *Shewanella putrefaciens* strains (29). Even though the TCA cycle may be complete under TMAO reduction conditions in MR-1, this pathway is probably only important for biosynthesis purposes (instead of energy production); there is very little carbon flux through the TCA cycle under anaerobic conditions due to the fact that most of the lactate taken up by the cell (>70%) is oxidized to pyruvate or acetate and secreted. This is consistent with the fact that the activities of some key enzymes in the TCA cycle (such as 2-ketoglutarate dehydrogenase) have been found to be much lower under anaerobic conditions than under aerobic conditions (32).

The majority of serine (81%) and glycine (65%) molecules were labeled at the carboxyl group, consistent with both amino acids being derived mainly from phosphoglycerate (via phosphoenolpyruvate). Since pyruvate had a larger fraction of its carboxyl groups labeled (96%), serine's precursor, phosphoenolpyruvate, may be derived not only from pyruvate but also from oxaloacetate (via OXA↔PEP + CO_2) or another source. The result, again, is consistent with a previous report that malate dehydrogenase (or malic enzyme) and PEP carboxylase (pyruvate + CO_2↔MAL) may be involved in this futile cycle (pyruvate + CO_2↔MAL↔OXA↔PEP + CO_2↔pyruvate) under anaerobic conditions (32).

Under fumarate reduction conditions, pyruvate was almost entirely (93%) labeled at its carboxyl group (Table 3). The labeling pattern of phenylalanine (using precursors of PEP and erythrose-4-phosphate) was similar to that found under TMAO-reducing conditions, which indicates that the pentose phosphate, Entner-Doudoroff (ED), and EMP pathways were mostly unchanged under the two anaerobic conditions. Less glutamate (56% versus 80%) was unlabeled under fumarate-reducing conditions than under TMAO-reducing conditions, with nearly 100% of the labeled glutamate having the label at the α-carboxyl group. Much more succinate was unlabeled under fumarate-reducing conditions than under TMAO-reducing conditions (95% versus 84%) because the large amount of unlabeled fumarate added as an electron acceptor was reduced to succinate (Table 1). Less aspartate and methionine were unlabeled under fumarate-reducing conditions (17% and 22%, respectively) than under TMAO-reducing conditions (58% and 64%, respectively). Therefore, the unlabeled fumarate was not incorporated into the TCA cycle and did not dilute the ^{13}C label, i.e., the unlabeled fumarate in the medium served only as an electron acceptor. Such observations can be explained by MR-1's very unique fumarate reductase (soluble, unidirectional, and periplasmic), in contrast to the membrane-

TABLE 1. Measured concentrations of metabolites under TMAO or fumarate reduction conditions ($n = 2$)[a]

Substrate (concn [mM])	Lactate concn (mM) in the medium	Metabolite concn (mM [mg/liter for glycine])[d]					Cell OD_{600}[d]	Dried biomass (g/liter)[c]
		Lactate	Glycine	Acetate	Pyruvate	Succinate		
TMAO (30)[b]	20	1.5 ± 0.5		6.6 ± 1.0	7.8 ± 2.2	0.4 ± 0.2	0.08 ± 0.01	~0.06
	22 (with 100 mg/liter glycine)	3.9 ± 0.5	53 ± 12	11.6 ± 0.3	3.6 ± 1.1	<0.1	0.11 ± 0.01	~0.08
Fumarate (23)	21	~0		13.2 ± 0.5	2.6 ± 1.3	22.0 ± 1.3	0.10 ± 0.01	~0.07
	20 (with 100 mg/liter glycine)	~0	37 ± 9	12.5 ± 0.7	1.9 ± 0.8	23.8 ± 1.0	0.14 ± 0.02	~0.10

[a] Samples without glycine were taken after 2 days of incubation, and samples with glycine were taken after 1 day of incubation.
[b] For TMAO culture, 100 mM NaCl was added to the medium to enhance growth.
[c] Dried cell weight was estimated by heating the biomass to constant weight at 95°C.
[d] Data are means ± standard deviations.

TABLE 2. Mass fragment distributions of key metabolites from *S. oneidensis* MR-1 growing under TMAO reduction conditions ($n = 2$)[a]

Metabolite	Fragment	Mass fraction				^{13}C-enriched position(s)[b]
		M0	M1	M2	M3	
Glycine	No loss	0.35	0.65	0	0	C-C*-OOH (first carbon)
	Loss of COOH	ND	ND	ND	ND	
Serine	No loss	0.19	0.81	0	0	C-C-C*-OOH (first carbon)
	Loss of COOH	0.99	0.01	0		
Glutamate	No loss	0.80	0.15	0.03	0.02	C-C-C-C-C*-OOH (first carbon)
	Loss of COOH	0.99	0.01	0	0	
Aspartate	No loss	0.58	0.35	0.06	0.01	C*-C-C-C*-OOH (first or fourth carbon or both carbons)
	Loss of COOH	0.78	0.19	0.02	0.01	
Methionine	No loss	0.64	0.27	0.10	0	C-S-C*-C-C-C*-OOH (first or fourth carbon or both carbons)
	Loss of COOH	0.78	0.17	0.05	0	
Phenylalanine	No loss	0.18	0.03	0.19	0.60	Carboxyl group is mostly labeled
	Loss of COOH	0.20	0.14	0.63	0.03	
Pyruvate	No loss	0.02	0.96	0.02	0	C-C-C*-OOH (first carbon)
	Loss of COOH	0.98	0.02	0		
Succinate	No loss	0.84	0.11	0.04	0.01	C-C-C-C*-OOH (first or fourth carbon [i.e., carboxyl group])
	Loss of COOH	0.90	0.06	0.03	0.01	

[a] BSTFA was used to derivatize organic acids, and TBDMS was used to derivatize amino acids. The variance in mass fractions for two duplicates was <10%. ND, none detected. ^{13}C was not enriched at the second and third positions of aspartate based on our further nuclear magnetic resonance measurement.
[b] *, ^{13}C-enriched position.

bound fumarate reductases found in other bacteria (20, 24, 31). In this case, fumarate reduction occurs mainly in the periplasm, and succinate is then primarily secreted into the medium rather than being transported through the cytoplasmic membrane, where it would be expected to enter the TCA cycle and complicate isotopomer analysis (for example, *Geobacter sulfurreducens* would transport fumarate into the cell and incorporate it into the TCA cycle during its reduction) (3).

The increased labeling in amino acids derived from the TCA cycle under fumarate-reducing conditions compared to that under TMAO-reducing conditions was further evidence that flux through the anapleurotic reactions increased and that the TCA cycle is branched (succinate is not produced from 2-ketoglutarate to supply OAA, but rather OAA is produced using anapleurotic reactions from the highly labeled pyruvate to provide amino acids and other metabolites in the TCA cycle). The evidence of a branched TCA cycle is consistent with the global transcriptome of MR-1 exposed to different electron acceptors. Genes encoding 2-ketoglutarate dehydrogenase displayed significant down-regulation under fumarate-reducing conditions compared to their expression under nitrate- or oxygen-reducing conditions (1).

A most surprising observation was the labeling pattern observed for serine and glycine under both TMAO and fumarate reduction conditions. According to the postulated serine-isocitrate lyase pathway (Fig. 1), formate (produced from the labeled carboxyl group in pyruvate) should be converted to formaldehyde and then incorporated into serine by condensation with glycine. If this were correct, then the third carbon of serine (hydroxyl conjugated) should be labeled. However, the MS data for both TMAO and fumarate reduction conditions clearly indicate that serine was almost entirely (~99%) labeled

TABLE 3. Mass fragment distributions of key metabolites from *S. oneidensis* MR-1 growing under fumarate reduction conditions ($n = 2$)[a]

Metabolite	Fragment	Mass fraction				^{13}C-enriched position(s)[b]
		M0	M1	M2	M3	
Glycine	No loss	0.19	0.81	0	0	C-C*-OOH (first carbon)
	Loss of COOH	ND				
Serine	No loss	0.10	0.88	0.01	0.01	C-C-C*-OOH (first carbon)
	Loss of COOH	0.99	0.01	0	0	
Glutamate	No loss	0.56	0.44	0	0	C-C-C-C-C*-OOH (first carbon)
	Loss of COOH	0.98	0.02	0	0	
Aspartate	No loss	0.17	0.43	0.39	0	C*-C-C-C*-OOH (first or fourth carbon or both carbons)
	Loss of COOH	0.49	0.50	0.01	0	
Methionine	No loss	0.22	0.36	0.37	0.01	C-S-C*-C-C-C*-OOH (first or fourth carbon or both carbons)
	Loss of COOH	0.50	0.44	0.04	0.02	
Phenylalanine	No loss	0.17	0.03	0.17	0.62	Carboxyl group is mostly labeled
	Loss of COOH	0.17	0.14	0.64	0.03	
Pyruvate	No loss	0.04	0.93	0.02	0.01	C-C-C*-OOH (first carbon)
	Loss of COOH	0.96	0.01	0.03	0	
Succinate	No loss	0.95	0.04	0.01	0	Not enriched
	Loss of COOH	0.95	0.04	0.01	0	

[a] BSTFA was used to derivatize organic acids, and TBDMS was used to derivatize amino acids. The variance in mass fractions for two duplicates was <10%. ND, none detected. ^{13}C was not enriched at the second and third positions of aspartate based on our further nuclear magnetic resonance measurement (unpublished data).
[b] *, ^{13}C-enriched position.

TABLE 4. Effects of nonlabeled glycine or glyoxylate on mass fragment distribution ([M-57] [no loss]) of key metabolites from *S. oneidensis* MR-1 growing under TMAO/fumarate reduction conditions ($n = 2$)[a]

Metabolite	Fragment	Mass fraction with substrate			Change of the labeling pool
		TMAO (Glycine)	Fumarate (Glycine)	Fumarate (Glyoxlate)	
Glycine	M0	0.85	0.67	0.43	Diluted
	M1	0.15	0.33	0.57	
Serine	M0	0.37	0.38	0.17	Diluted
	M1	0.63	0.61	0.80	
	M2	0	0.01	0.02	
Glutamate	M0	0.77	0.56	0.54	Not diluted
	M1	0.18	0.43	0.45	
	M2	0.03	0.01	0.01	
Aspartate	M0	0.55	0.17	0.20	Not diluted
	M1	0.33	0.40	0.41	
	M2	0.10	0.43	0.38	
Phenylalanine	M0	0.17	0.16	0.15	Not diluted
	M1	0.04	0.02	0.02	
	M2	0.21	0.17	0.18	
	M3	0.57	0.63	0.62	

[a] The variance in mass fractions for two duplicates was <10%.

at its carboxyl group. This observed labeling pattern would be expected if serine was synthesized from pyruvate (pyruvate→PEP→serine). This hypothesis is further strengthened by considering the nearly identical isotope enrichment patterns of methionine and aspartate under anaerobic conditions. The majority of the carbon in both amino acids is derived from the same precursor (oxaloacetate), but methionine obtains an additional carbon from the C_1 pool. This indicates that the C_1 pool, in the form of N^5,N^{10}-methyl H_4 folate, was not labeled either (8). Since the C_1 pool derives mainly from the second carbon of glycine and the third carbon of serine via the reactions serine→C_1 + Gly and Gly→C_1 + CO_2 (Fig. 2c), this result provides further evidence that these carbons were indeed unlabeled. Thus, since the serine-isocitrate lyase pathway introduces the labeled carbon from the carboxyl group of pyruvate into the third position of serine (Fig. 1), the absence of ^{13}C both in fragmented serine molecules that have lost their carboxyl groups and in carbon originating from the C_1 pool generally contradicts the previously proposed serine-isocitrate lyase pathway (32).

There are two other reasons that the serine pathway may not be used generally under all anaerobic conditions for *S. oneidensis* species. First, the isotope fraction in *Shewanella algae* lipids from cells grown under iron-reducing conditions supports the use of the serine-isocitrate lyase pathway rather than

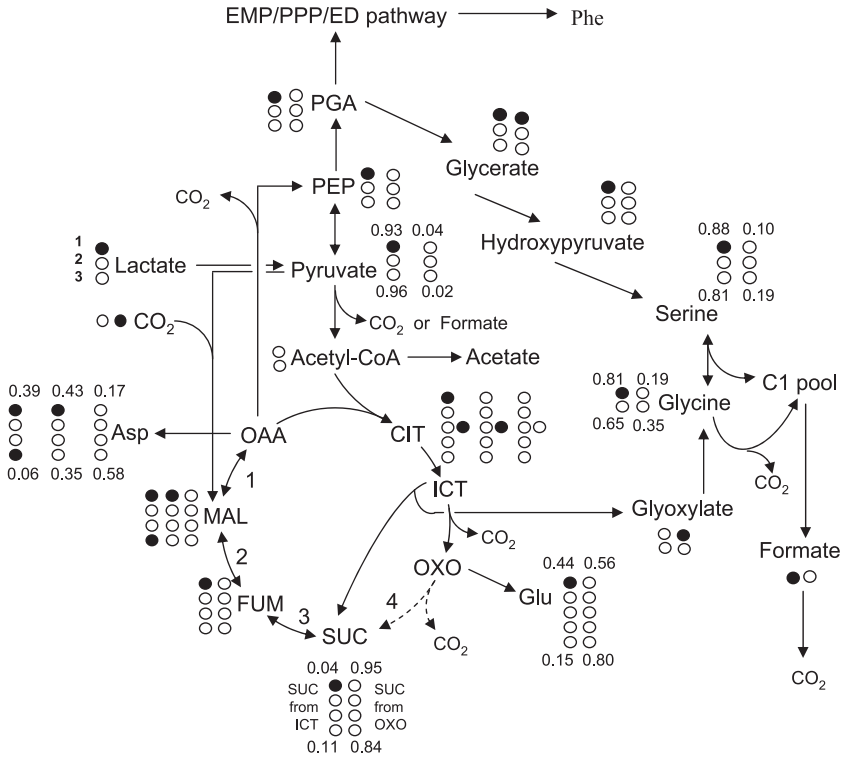

FIG. 4. Anaerobic pathway under TMAO and fumarate reduction conditions, determined via isotopomer path tracing. The black dots denote the possible ^{13}C-enriched positions in the key metabolites, and the white dots denote the carbon positions not enriched with ^{13}C. The numbers below the dots are the fractions of the particular metabolites under TMAO reduction conditions, and the numbers above the dots are the fractions of the metabolites under fumarate reduction conditions. The proposed pathway model contains a serine degradation route and a TCA cycle (containing two different conditions). Under TMAO reduction conditions, net flux was through a complete TCA cycle (succinate originating from 2-ketoglutarate would not be expected to be labeled, while succinate originating from isocitrate would be expected to be labeled on its carboxyl group); under fumarate reduction conditions, there are two anapleurotic fluxes (OXA→fumarate→succinate and citrate→OXO), and the existence of a complete TCA cycle is in question given the lack of evidence for an OXO→succinate reaction. See the legend to Fig. 1 for abbreviations.

other pathways, but the isotopic data for lipids from *S. oneidensis* MR-4 (a species with a very similar phenotype to that of MR-1) (37) do not support the use of the serine pathway for this strain grown under nitrate-reducing conditions (35, 40). Second, although the serine pathway might be used during growth on formate as the sole source of carbon and energy, it is not energetically efficient (consuming ATP and NADH) at transforming pyruvate to a one-carbon compound (formate) and then using the formate to synthesize multicarbon compounds via the serine pathway when lactate and pyruvate are available.

Serine and glycine had larger labeled fractions under fumarate-reducing conditions (88% and 81%, respectively) than under TMAO-reducing conditions (81% and 65%, respectively). Under both conditions, all of the label in serine was at the carboxyl group, which is transferred to glycine if glycine originates from serine. However, the labeling ratio for serine to glycine was <1, especially under TMAO reduction conditions, which indicates that part of the glycine may be synthesized from glyoxylate (Fig. 1). Although the genome annotation of *S. oneidensis* MR-1 (www.microbesonline.org) indicates that this organism does not have the genes encoding either serine-glyoxylate transaminase or glycine transaminase (needed for the glyoxylate-to-glycine transformation in the proposed serine-isocitrate lyase pathway), new genes encoding serine-glyoxylate transaminase have been reported recently (33). This finding is supported by the isotopic data presented here. For example, under fumarate reduction conditions, the labeled glycine pool in the biomass was significantly diluted when the culture was supplemented with 100 mg unlabeled glyoxylate (Table 4); however, the labeled aspartate and glutamate pools were nearly intact (changes were below the measurement noise). This indicates that most unlabeled glyoxylate did not enter the TCA cycle but was converted to glycine and serine.

The glycine metabolism route (Fig. 2c) was further tested by providing a source of unlabeled glycine (100 mg/liter, i.e., 1.3 mM) in the medium under fumarate or TMAO reduction conditions (Table 4). Even with glycine available, there was no evidence for the transformation of the carboxyl carbon of pyruvate to the third position of serine under either the fumarate-reducing conditions (Fig. 2) or the TMAO-reducing conditions (data not shown). Furthermore, the labeled pools of PEP (precursor of phenylalanine), OAA (precursor of aspartate), and OXO (precursor of glutamate) did not change significantly after unlabeled glycine was added, i.e., the unlabeled glycine was not incorporated into the central metabolic pathway but, more likely, was degraded. Meanwhile, much less serine was observed to be labeled, suggesting that the reaction between serine and glycine is highly reversible (30) but not able to incorporate labeled formaldehyde. Glycine and serine additions enhanced growth under anaerobic conditions, but neither glycine nor serine can serve as a sole carbon source (without the addition of lactate) to support anaerobic growth of MR-1 (unpublished data) or other *Shewanella putrefaciens* strains (29). This also suggests that MR-1 may not be able to convert serine and glycine to all central metabolites via the proposed serine pathway (Fig. 1) for biosynthesis or energy production (ATP and NADH) (27).

To reconcile the isotope data with reported enzyme activities and the annotated genome sequence, we propose a modified anaerobic metabolic network for *S. oneidensis* MR-1 growing with lactate in minimal medium (Fig. 4). The anaerobic network contains a serine degradation route and anapleurotic reactions encompassing malate, pyruvate, PEP, and oxaloacetate. Although most of the lactate (>70%) was converted to acetate and pyruvate, MR-1 appears to have a complete TCA cycle under TMAO-reducing conditions. In addition, MR-1 appears to be able to efficiently convert intracellular pyruvate into serine via the reactions pyruvate→GAP→hydroxypyruvate→serine (based on the reported high activity of hydroxypyruvate reductase) (32) and to degrade it via the reactions glycine→C_1 pool→formate→CO_2 (consistent with the reported high activity of formate dehydrogenase) (32). Medium supplementation with glycine demonstrated that the conversion of serine to glycine is highly reversible and that even under favorable conditions, there is no incorporation of labeled formaldehyde into serine. This evidence, as well as that in previous studies (29, 32), suggests that the degradation of serine and glycine may be an important pathway for MR-1 growth under anaerobic conditions. As a bacterium involved in putrefaction in ocean sediments (which could be protein-rich) (2, 22), *S. oneidensis* MR-1 may have evolved to favor the degradation of carbon compounds via amino acid catabolic pathways under anaerobic conditions, when the TCA cycle is less effective.

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